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Modulation of Crayfish Hearts by FMRFamide-related Peptides

A. JOFFRE MERCIER[1] AND RUNE T. RUSSENES

Department of Biological Sciences, Brock University, St. Catharines, Ontario L2S 3A1 Canada

Abstract. The present study examines effects of FMRFamide-related peptides (FaRPs) on crayfish heart. Lobster peptides F_1 (TNRNFLRFamide) and F_2 (SDRNFLRFamide) increase the rate and amplitude of heart beat in hearts isolated from *Procambarus clarkii*. Thresholds for these effects were between 10^{-10} and 10^{-9} *M* for F_2 and between 10^{-9} and 10^{-8} *M* for F_1. FMRFamide and FLRFamide elicited similar cardioexcitatory effects, but at thresholds of approximately 10^{-7} *M*. Thus, the amino-terminal extensions "TNRN" and "SDRN" enhance the excitatory actions of FMRFamide and FLRFamide. SchistoFLRFamide (PDVDHVFLRFamide) and leucomyosuppressin (pQDVDHVFLRFamide) markedly decrease the rate of cardiac contractions at 10^{-9} to 10^{-8} *M* and can suppress the cardiac rhythm for one minute or more at 10^{-7} *M*. The amino-terminal extensions of these two peptides, therefore, are necessary for inhibition of heart rate. Both of these peptides cause an initial reduction in contraction amplitude, but contractions subsequently increase in the presence of schistoFLRFamide. Thus, crayfish hearts are sensitive to several FMRFamide-related peptides, but the sites and mechanisms of action remain to be determined.

Introduction

Since the discovery of the neuropeptide FMRFamide (Phe-Met-Arg-Phe-NH_2) in the bivalve mollusk *Macrocallista nimbosa* (Price and Greenberg, 1977), FMRFamide-related peptides (FaRPs) have been reported in numerous invertebrate and vertebrate species (*e.g.*, Boer *et al.*, 1980; Dockray *et al.*, 1983; Watson *et al.*, 1984; Grimmelikhuijzen and Graff, 1985; Lehman and Price, 1987; Li and Calabrese, 1987; Elphick *et al.*, 1989; Robb *et al.*, 1989; Krajniak and Price, 1990). It is now recognized that FaRPs represent members of a very large family of neuropeptides that is widely distributed throughout the animal kingdom (Greenberg and Price, 1983; Price and Greenberg, 1989).

In crustaceans, FMRFamide-like immunoreactivity (FLI) has been found throughout most of the nervous system, but the highest amounts are present in the pericardial organs (POs) (Koberski *et al.*, 1987; Marder *et al.*, 1987; Krajniak, 1991; Mercier *et al.*, 1991b). Because the POs are located in the pericardial sinus just outside the heart (Maynard, 1960), and because they release cardioactive hormones (*e.g.*, Cooke and Sullivan, 1982; Kravitz *et al.*, 1980), the heart is likely to be an important target for crustacean FaRPs. So far, only two FaRPs have been sequenced and identified in extracts of the lobster POs, although other FMRFamide-like immunoreactive material is present (Trimmer *et al.*, 1987). These two peptides have the sequences TNRNFLRFamide (F_1) and SDRNFLRFamide (F_2). Both of these peptides excite isolated hearts of lobsters (Kravitz *et al.*, 1987) and blue crabs (Krajniak, 1991). However, the effects of these and other FaRPs on crustacean hearts have not been thoroughly investigated.

The primary aim of the present study was to examine in greater detail the cardio-regulatory effects of lobster peptides F_1 and F_2. The effects of these two peptides were studied on isolated crayfish hearts. To examine the relationship between amino acid sequence and biological activity, the effects of F_1 and F_2 were compared with those of FMRFamide, FLRFamide, and two FaRPs with significantly different amino-terminal extensions: leucomyosuppressin (LMS), with the sequence pQDVDHVFLRFamide (Holman *et al.*, 1986) and SchistoFLRFamide (Sch), with the sequence PDVDHVFLRFamide

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[1] To whom correspondence should be addressed.

(Robb *et al.*, 1989). The crayfish heart was sensitive to all of the compounds tested.

Materials and Methods

Crayfish were obtained commercially and were maintained in aerated freshwater tanks at 14.5°C on a mixed vegetable diet.

Synthetic peptides were applied to spontaneously active crayfish hearts. The dorsal carapace, containing the heart and pericardium, was dissected from crayfish weighing approximately 3 g and was pinned to a Sylgard-lined dish with the ventral side up. The pericardial membrane was severed and pinned at each side to allow the bathing fluid access to the heart. The recording chamber, which had a volume of 0.5 ml, was perfused with crayfish saline (van Harraveld, 1936), which had the following constituents (in m*M*): Na^+, 205; Cl^-, 232; K^+, 5.3; Ca^{++}, 13.5; Mg^{++}, 2.5; HEPES, 5.0 (pH 7.4). Saline was added to the chamber at a rate of 3.0 ml min^{-1} using a peristaltic pump and was removed at the other end of the chamber by suction. The entire preparation was superfused continuously in this manner. The temperature was maintained at 14–16°C, but did not vary by more than 1°C during any one experiment. Heart preparations were viable for up to 8 h.

Contractions were recorded by connecting the sternal artery to a tension transducer using two insect pins that were hooked at one end and glued to the transducer at the other end. The artery and heart were stretched until the maximum contraction amplitude was obtained. Contractions were displayed on a Grass Model 7B Polygraph. The rate and amplitude of contractions were measured manually over intervals of either 30 s or 1 min.

Peptides were applied by changing the perfusate to a solution containing a selected peptide concentration. Peptides were present in the bathing chamber for 8–10 min; 10 min was chosen arbitrarily as the maximum time of exposure. In a few cases, it was obvious that the maximal effect of the peptide had already occurred, and washout was begun after 8 min. The maximal effect of the peptides generally occurred well before this time, except for the increased amplitude caused by SchistoFLRFamide (see Results).

During washout, the effects of the peptides began to subside within 5 min and had completely worn off within 20–30 min. In many cases (*e.g.*, Fig. 7), the effects wore off more rapidly. Each peptide was tested by starting with the lowest concentration (10^{-12} *M*) and subsequently increasing the dosage in 10-fold increments until the entire response range had been tested. Successive doses were always given after the previous dose was completely washed out. Three successive applications of 1×10^{-9} *M* F_2 onto the same preparation yielded virtually identical effects each time. The results reported here were obtained from a total of 37 preparations. In all but six experiments, each peptide was tested on one preparation only. The number of preparations used for testing each peptide is indicated in the figure legends.

SchistoFLRFamide and leucomyosuppressin were obtained from Peninsula Laboratories Ltd. (Belmont, California). Peptides F_1 and F_2 were synthesized by Dr. D. McCormack (Rochester, Minnesota) and were a gift from Dr. M. Schiebe. FMRFamide, FLRFamide, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri).

In each case, the error value reported is the standard error of the mean. Statistical significance of differences between mean values was determined using a Student's *t* Test (Furguson, 1971).

Results

Of the six neuropeptides tested, four elicited responses that were exclusively cardio-excitatory. FMRFamide, FLRFamide, F_1, and F_2 increased both the rate and amplitude of contractions of isolated *Procambarus* hearts. Figure 1 shows representative examples of the effects of these four peptides, at doses that elicited approximately equivalent responses. The onset of such responses usually

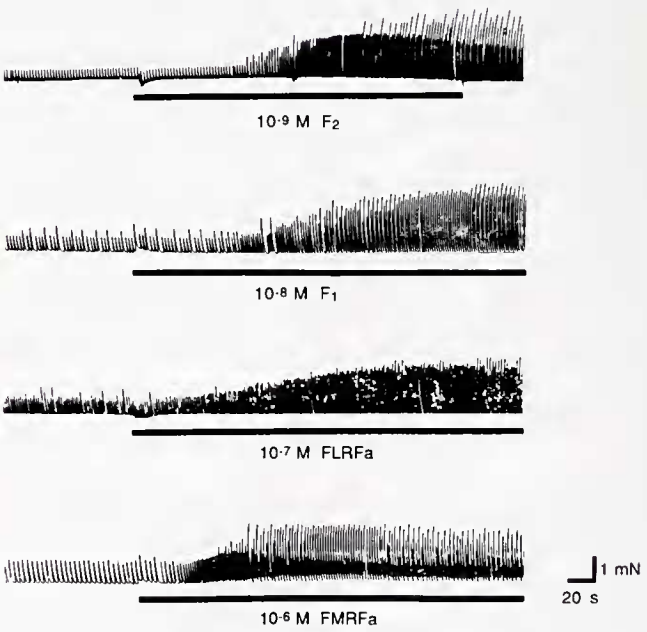

Figure 1. Effects of excitatory FaRPs on crayfish heart contractions. Each panel shows chart recordings of spontaneous contractions. Peptides, at the concentrations indicated, were present in the bathing solution during the periods indicated by the thick horizontal bars. The recordings were obtained from different preparations. (Abbreviations: FLRFa for FLRFamide, FMRFa for FMRFamide, F_1 and F_2 as in text.)

occurred within 60–90 s after the peptide entered the bathing chamber, and the lag-time was shorter at higher peptide concentrations. The effects on heart rate and contraction amplitude were always completely reversed by 20–30 min of washing in normal saline (data not shown). None of these four peptides elicited any inhibitory effects.

Log dose-response curves, constructed for the four excitatory peptides (Fig. 2), were based on the responses of five to six preparations in each case. Responses were expressed as the percentage change in contraction rate and the percentage change in amplitude by comparing the maximal effect of each peptide with the average contraction rate, or contraction amplitude, during the 3 min period immediately preceding peptide application. Peptides F_1 and F_2 caused a more pronounced increase in the amplitude of the contractions than on their rate. For FLRFamide and FMRFamide, however, the percentage change in amplitude was similar to the change in rate in each case.

Differences in the relative potencies of the peptides were more prominent for the effect on contraction amplitude (Fig. 2A) than for the effect on rate (Fig. 2B). A comparison of the effects on contraction amplitude gave the following results. F_2 was the most potent peptide, with a threshold concentration between 10^{-10} and 10^{-9} *M*. A comparison of the effects of 10^{-9} *M* F_2 and 10^{-5} *M* FMRFamide suggests that F_2 was up to 10,000 times more potent than FMRFamide. F_1 was the next most potent peptide, with a threshold between 10^{-9} and 10^{-8} *M*, and was approximately 1000 times more potent than FMRFamide (based on the effects of 10^{-8} *M* F_1 and 10^{-5} *M* FMRFamide). FLRFamide was about 10 times more potent than FMRFamide (based on the effects of 10^{-7} *M* FLRFamide and 10^{-6} *M* FMRFamide), but the threshold concentrations for both appeared to be between 10^{-8} and 10^{-7} *M*. FMRFamide gave a relatively broad log-dose *versus* response curve, which rose steadily over the concentration range of 10^{-8} to 10^{-5} *M*, while the other peptides appeared to reach saturation within slightly narrower concentration ranges.

Differences in relative potency were not as marked when comparing the effects of the excitatory peptides on contraction rate (Fig. 2B). F_2 and F_1 had approximately equivalent effects on heart rate, but both compounds were about 100 times more potent than FLRFamide and FMRFamide. The log-dose *versus* response curves for FLRFamide and FMRFamide were very similar.

SchistoFLRFamide (Sch) and leucomyosuppressin (LMS) had inhibitory effects on cardiac contractions at concentrations of 10^{-9} to 10^{-7} *M* (Figs. 3–5). The rate of spontaneous contractions was reduced consistently by both peptides. At 10^{-7} *M*, contractions were completely suppressed for a period lasting 1 min or longer, after which

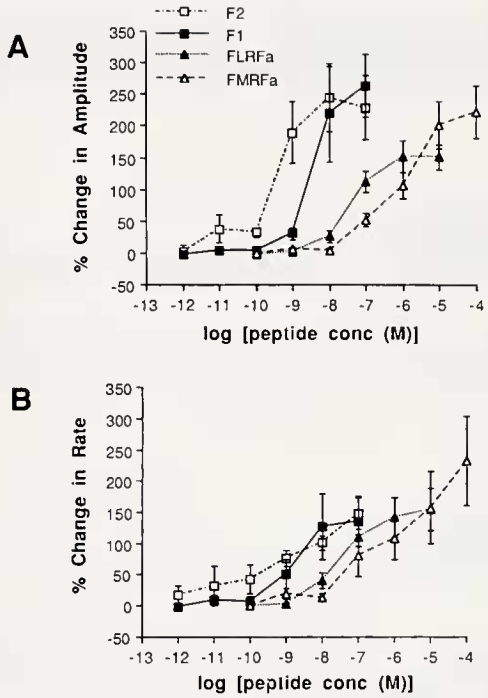

Figure 2. Log-dose *versus* response curves for the effects of excitatory FaRPs on the amplitude of cardiac contractions (A) and on heart rate (B). The percentage change in rate or in amplitude was determined by comparing the maximum value obtained in the presence of the peptide from the average value during the three minutes prior to peptide application:

$$\% \text{ change} = [(\text{peak value} - \text{initial value})/\text{initial value}] \times 100.$$

Each point represents the mean value for five preparations in the case of F_2 and for six preparations in all other cases. Error bars depict standard errors of the means. (Abbreviations are as in Fig. 1.)

contractions resumed at a rate that was lower than before peptide application (Figs. 3, 4, and 6A). Dose-response curves, based on the maximal reduction in rate, were markedly similar for these two peptides (Fig. 5A). The threshold for inhibition of heart rate was between 10^{-10} and 10^{-9} *M*.

Effects of Sch and LMS on the amplitude of contractions were more complex. For most preparations, such as the one represented in Figure 4, the contractions that persisted in the presence of either Sch or LMS were initially reduced in amplitude. This type of effect was observed in 4 of 6 preparations exposed to Sch and in 6 of 8 preparations exposed to LMS. Figure 3 is an example of recordings from a preparation in which no substantial reduction in contraction amplitude occurred during exposure to LMS. When observed, reductions in contraction size were usually transient. Approximately 2–4 min after peptide exposure began, 10^{-7} *M* Sch caused a substantial increase in contraction size above the level observed prior

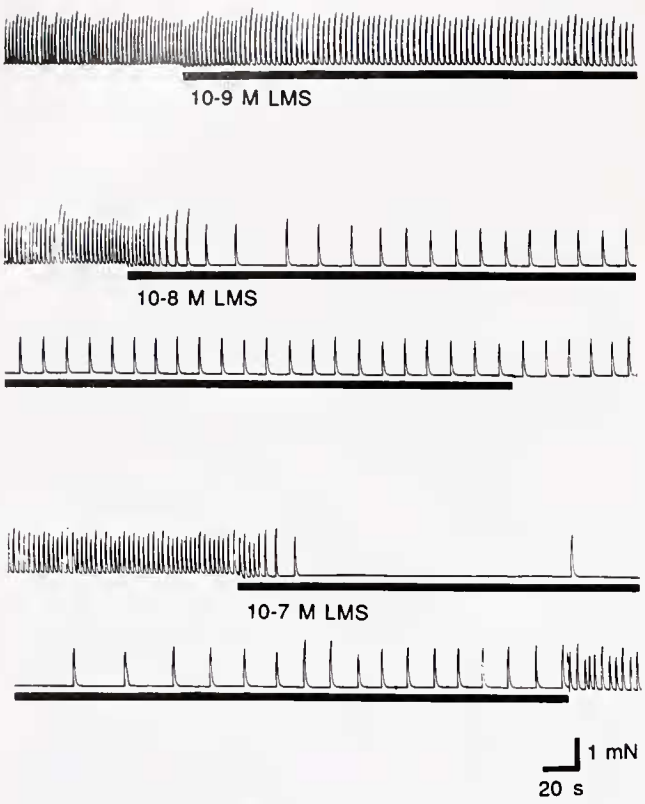

Figure 3. Effects of LMS on heart contractions. Each panel shows chart recordings of spontaneous cardiac contractions. LMS was present in the bathing solution during the periods indicated by the thick horizontal bars at the concentrations indicated. The recordings were all from the same preparation.

to peptide exposure (Fig. 4). Thus, the effect of Sch on contraction amplitude appeared to be biphasic.

Log-dose *versus* response curves for the initial effect of LMS and Sch on contraction amplitude were obtained by comparing responses 1 min after the peptide entered the bath with the average amplitude over a 2-min period prior to peptide exposure (Fig. 5B). (The change in contraction size was expressed as a percentage of the amplitude during the period prior to peptide application.) The dramatic reduction in contraction size at 10^{-7} *M* was due mainly to the fact that this dose completely suppressed contractions in most preparations. Log-dose *versus* response curves for the increase in amplitude that occurred later were obtained by comparing the average contraction amplitude during the 2-min period before peptide application with the highest average amplitude over a 1-min period during exposure to the peptide or during the first 5 min of the wash-out period (Fig. 5C). On average, contraction amplitudes doubled in 10^{-8} *M* Sch and tripled in 10^{-7} *M* Sch. In contrast, 10^{-7} *M* LMS caused only a 25% increase in contraction size.

The increase in contraction amplitude caused by Sch developed more slowly than did the reduction in contraction rate. Figure 6A shows the time course of the effects of 10^{-8} *M* Sch for an individual preparation. In this case, heart rate began to decline within 2 min of peptide exposure and reached its lowest value 6 min later. Contraction amplitude, however, did not begin to rise until 6 min after the peptide entered the bath and required an additional 8 min to reach its maximal level. In experiments with six preparations exposed to 10^{-7} *M* Sch, the mean time for the maximal inhibition of heart rate (1.3 ± 0.36 min) was shorter than the mean time for the maximal increase in amplitude (10.9 ± 2.0 min), and the difference in means was statistically significant ($t = 5.56$; $P < 0.01$).

In contrast, the increased amplitude caused by F_2 occurred rapidly and usually coincided with the maximal increase in rate, as in the example shown in Figure 6B. Mean times for the maximal rate (2.7 ± 0.68 min) and amplitude (3.1 ± 0.99) for five preparations were not significantly different ($t = 0.93$; $P > 0.4$). As in the example shown in Figure 6B, the heart rate often declined rapidly from the peak value, even though F_2 was still present in the bathing solution. Contraction amplitude, however, remained elevated until the peptide was removed.

As a first step toward examining potential interactions between FaRPs, the effect of a mixture of F_2 and Sch was studied. We were particularly interested in determining

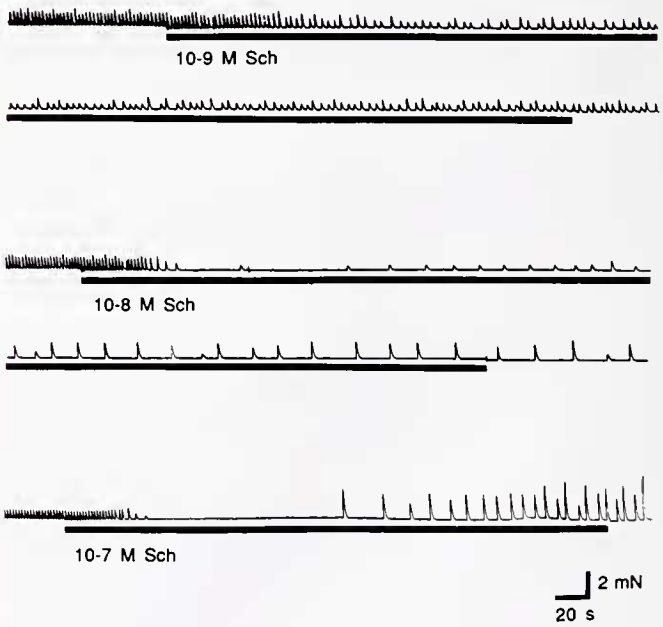

Figure 4. Effects of Sch on heart contractions. Each panel shows chart recordings of spontaneous cardiac contractions. Sch was present in the bathing solution during the periods indicated by the thick horizontal bars at the concentrations indicated. The recordings were all from the same preparation.

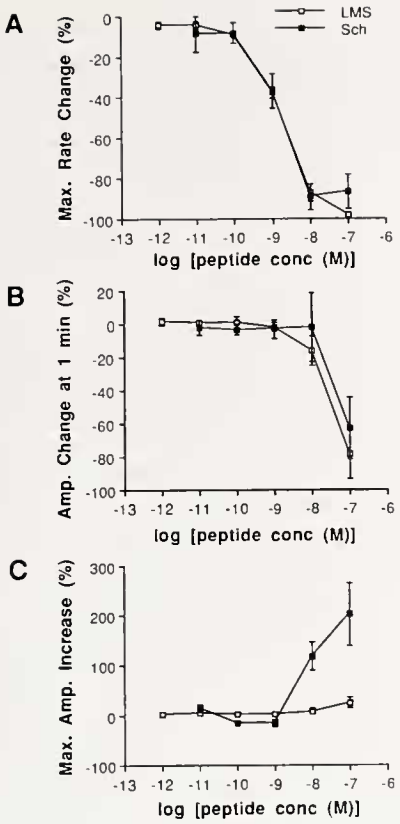

Figure 5. Log-dose *versus* response curves for effects of LMS and Sch on heart rate (A), on the amplitude of contractions after 1 min of peptide exposure (B) and on the maximum amplitude of contractions during peptide exposure or in the first 5 min of the wash-out period (C). The change in rate or in amplitude was determined by comparing the value obtained in response to the peptide with the average value prior to peptide application and was expressed as a percentage of the initial value as in Figure 2. Effects on heart rate (A) were determined using the minimum heart rate during peptide exposure. Each point represents the mean value obtained from eight preparations exposed to LMS and from six preparations exposed to Sch. Error bars depict standard errors of the means.

whether the chronotropic action of one peptide might predominate or, alternatively, whether the two substances might produce an "additive" response. Figure 7 illustrates the responses of an individual preparation in which 10^{-8} M F_2 caused a rapid 60% increase in heart rate and 10^{-8} M Sch decreased heart rate by about 50%. A mixture of the two peptides produced a heart rate that increased only slightly and was comparatively stable. Subsequent application of F_2 showed that the heart was capable of responding to this peptide as before. Thus, these two peptides exert chronotropic actions that are mutually antagonistic and tend to cancel each other out when combined. Sch did not antagonize the effect of F_2 on contraction size and may even have potentiated it (Fig. 7). This experiment was performed six times with qualitatively similar results

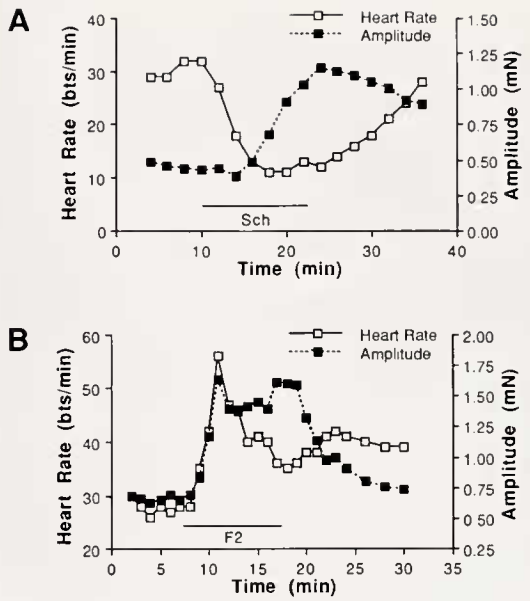

Figure 6. Time course for the effects of Sch (A) and F_2 (B) on the rate and amplitude of cardiac contractions. The peptides were present in the bathing solution at concentrations of 10^{-8} M during the periods indicated by the horizontal bars. The data for (A) and (B) were obtained from the same experimental preparation.

in each case. Sch always antagonized the chronotropic effect of F_2 but not its inotropic effect. Thus, while F_2 is chronotropically and inotropically excitatory, and Sch is chronotropically inhibitory, a mixture of these FaRPs can produce a response that is predominantly inotropic.

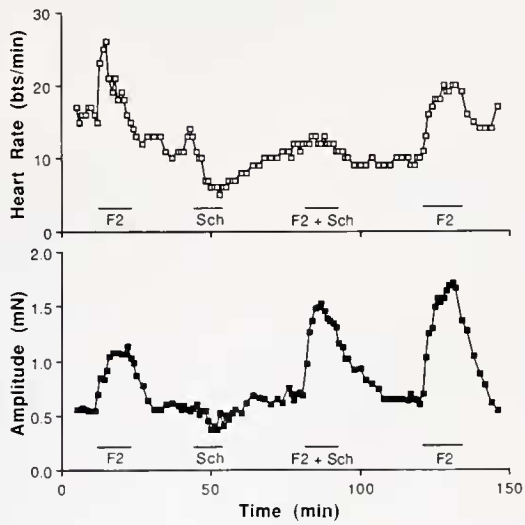

Figure 7. The effect of a mixture of F_2 and Sch on the rate (upper panel) and amplitude (lower panel) of cardiac contractions. The data were obtained from a single preparation. Peptides were present in the bathing solution at 10^{-8} M at the times indicated by the horizontal bars.

Discussion

FaRPs have been reported to modulate the activity of hearts from several types of invertebrates, including mollusks (*e.g.*, Painter and Greenberg, 1982; Price *et al.*, 1990), insects (Cuthbert and Evans, 1989; Robb *et al.*, 1989), leeches (Li and Calabrese, 1987), and crustaceans (Kravitz *et al.*, 1987; Krajniak, 1991). Some FaRPs elicit responses that are mainly excitatory, others elicit responses that are primarily inhibitory, and others are reported to elicit mixed or biphasic responses (Cuthbert and Evans, 1989). The type of response depends on the chemical structure of the peptide and on the invertebrate species (Painter and Greenberg, 1982).

The present study did not investigate the sites or mechanisms of action of the various FaRPs tested. Changes in rate are most likely to result from effects on the cardiac ganglion, which sets the overall rhythm for cardiac contractions (Maynard, 1960). Changes in the amplitude of contractions could be due to changes in the number or frequency of nerve impulses per burst produced in the cardiac motor neurons (Maynard, 1960), or they could result from direct effects on the cardiac muscle cells, as in *Limulus* (Watson *et al.*, 1985).

It should be noted, however, that the POs, which contain several cardioexcitatory agents (*e.g.*, Cooke and Sullivan, 1983), were still present in the preparations used for study. We cannot exclude the possibility that the applied peptides act by inducing the release of other agents from the POs. Such hormone-induced release of hormones from the same neurosecretory organ has not been reported for any of the pericardial substances and seems unlikely. In addition, the inhibitory effects of Sch and of LMS are qualitatively similar to those reported in insect hearts, which lack POs (Cuthbert and Evans, 1989; Robb *et al.*, 1989).

F_1 and F_2 exert purely excitatory effects on hearts of *Procambarus*, increasing both the rate and amplitude of spontaneous contractions. F_1 and F_2, respectively, are up to 1,000 and 10,000 times more potent than FMRFamide when comparing inotropic responses. Similar results have been obtained with isolated hearts of the blue crab, *Callinectes sapidus* (Krajniak, 1991) and of the lobster, *Homarus americanus* (Kravitz *et al.*, 1987). F_1 is also 1000 times more potent than FMRFamide in exciting the semi-isolated heart of the locust, *Schistocerca gregaria* (Cuthbert and Evans, 1989). Thus, arthropod hearts appear to be more sensitive to N-terminally extended analogues of FLRFamide than to FMRFamide. FMRFamide and FLRFamide are both excitatory, but the addition of the amino acid sequence thr-asn-arg-asn- or of ser-asp-arg-asn- to the amino terminal of the tetrapeptide-phe-leu-arg-phe-NH_2 effectively increases the potency of the peptide. Similar structure-activity relationships have been reported for the locust extensor tibiae nerve-muscle preparation (Cuthbert and Evans, 1989) and for neuromuscular synapses on crayfish abdominal extensor muscles (Mercier *et al.*, 1990).

The sensitivity of hearts from *Homarus* (Kravitz *et al.*, 1987), *Callinectes* (Krajniak, 1991), and *Procambarus* to F_1 suggests that F_1 or closely related peptides are common cardioexcitatory hormones in decapods. F_1 and F_2 were originally isolated from lobster POs (Trimmer *et al.*, 1987) and have not been positively identified in any other tissues to date. The POs of *Procambarus clarkii*, however, appear to contain cardioexcitatory FaRPs that are very similar to F_1 and F_2 (Mercier *et al.*, 1991a, b).

The crayfish heart was also sensitive to Sch and LMS, which have markedly different N-terminal extensions than the other FaRPs studied. The primary effect of Sch and of LMS appears to be a reduction in heart rate, which occurs at a threshold concentration between 10^{-10} and 10^{-9} *M* for both peptides. Similar thresholds have been reported for inhibition of locust hearts (Robb *et al.*, 1989) and oviducts (Lange *et al.*, 1991) by Sch, and for inhibition of cockroach hindguts (Holman *et al.*, 1986) and locust hearts (Cuthbert and Evans, 1989) by LMS. Because FLRFamide excites the crayfish heart, the inhibitory effects observed in the present study must be due to the presence of the N-terminal extensions PDVDHV- and pQDVDHV- on Sch and LMS, respectively.

In contrast, Krajniak (1991) has reported that LMS causes cardioexcitation in *Callinectes*. The effect was observed in a single preparation and has a threshold 10,000 times higher than that of F_1. Such an observation might reflect species-dependent differences in the target tissues, but this possibility requires further study.

Reduction in the amplitude of contractions was not observed as consistently with crayfish hearts as with insect preparations (Cuthbert and Evans, 1989; Lange *et al.*, 1991). The large increase in contraction size at higher Sch concentrations was distinct from the inhibitory effect in that it developed much more slowly. In addition, the large increase in amplitude was only produced by Sch, whereas both Sch and LMS could inhibit heart rate. This indicates that Sch activates a receptor that is not activated by LMS. This might seem surprising, in view of the high degree of homology between the two peptides, which differ by only the N-terminal amino acid. It is possible, however, that at the low concentrations, both peptides activate the same receptor or receptor class (one responsible for reducing heart rate), and that a different class of receptor is activated at higher concentrations of Sch (causing the increase in amplitude). This hypothesis is consistent with the high similarity between the log-dose *versus* response curves for the effects of Sch and LMS on contraction rate (Fig. 5A).

The reason for the delayed inotropic effect of Sch is not clear. One possibility may be that the peptide is hydrolyzed beginning at the N-terminal end. This would gradually produce a peptide more closely resembling FLRFamide, which, at 10^{-7} *M*, caused a substantial increase in contraction size (Figs. 1, 2). The combination of Sch with an excitatory FaRP (F_2) was capable of increasing the amplitude of contractions without increasing heart rate (Fig. 7). Thus, if hydrolysis were to cause an accumulation of an excitatory FaRP, the combination of Sch and its breakdown product would be expected to produce a response similar to that observed after several minutes of exposure to Sch (Figs. 4, 6A). Such an explanation, however, is speculative, and others are possible.

Responses to mixtures of excitatory and inhibitory agents may be of some physiological significance. Increases in cardiac output (the fluid volume ejected by the heart per minute) during physical activity (*e.g.*, McMahon *et al.*, 1979) and hypoxia (*e.g.*, Burnett, 1979; McMahon and Wilkens, 1975) generally involve changes in both heart rate and stroke volume (the amount of fluid ejected per beat). In some cases, however, stroke volume increases independently of heart rate (Taylor, 1976; McMahon and Wilkens, 1977; Taylor and Butler, 1978), and the mechanisms that underly such an effect are not known (McMahon and Burnett, 1990). It is tempting to speculate that a mixture of peptides like F_2 and Sch, which is capable of increasing the amplitude of contractions independently of rate, might be involved. Tension recordings, however, do not provide a direct measure of stroke volume or of cardiac output. In addition, crayfish POs do not contain Sch or LMS (Mercier *et al.*, 1991b), and none of the neurohormones isolated from POs to date decreases heart rate. More research aimed at identifying neuropeptides and examining their effects may provide a better understanding of how stroke volume and cardiac output are regulated.

Acknowledgments

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